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In re application of

David N. COOPER et al.

Serial No. 09/853,688

GROUP 1623

Filed May 14, 2001

METHOD FOR DETECTING GROWTH  
HORMONE VARIATIONS IN HUMANS,  
THE VARIATIONS AND THEIR USES

**CLAIM FOR FOREIGN PRIORITY UNDER 35 U.S.C. 119  
AND SUBMISSION OF PRIORITY DOCUMENT**

Commissioner of Patents

Washington, D.C. 20231

Sir:

Attached hereto are certified copies of applicants' corresponding patent applications filed in Great Britain on May 12, 2000, under No. 0011459.5 and in Europe on July 14, 2000, under No. 00306004.3.

Applicants herewith claim the benefit of the priority filing dates of the above-identified applications for the above-entitled U.S. application under the provisions of 35 U.S.C. 119.

Applicants inadvertently indicated that the filing date of European Application 00306004.3 was May 12, 2000. The correct priority date should be July 14, 2000, as can be seen from the second page of the European priority document attached hereto.

Applicants also attach hereto a corrected copy of the Application Data Sheet reflecting the correct European

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filing date. Please update the USPTO patent data base to reflect the above change.

Respectfully submitted,

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November 1, 2001



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3. Full name, address and postcode of the or of each applicant (underline all surnames)	University of Wales College of Medicine Heath Park, Cardiff CF14 4XN		
Patents ADP number (if you know it)	0049854.6001		
If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom		
4. Title of the invention	SEQUENCES		
5. Name of your agent (if you have one)	Wynne-Jones, Laine & James		
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Signature \_\_\_\_\_ Date 12.5.00

Wynne-Jones, Elaine & James

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## SEQUENCES

The present invention relates to a method for detecting naturally-occurring growth hormone mutations; to mutations thereby detected and their use in screening patients  
5 for growth hormone irregularities or for producing variant proteins suitable for treating such irregularities.

That human stature was influenced by inherited factors was understood more than a century ago. Although familial short stature, with its normally recessive mode of  
10 inheritance, was recognised as early as 1912, it was a further quarter century before such families came to be properly documented in the scientific literature. The recognition that recessively inherited short stature was commonly associated with isolated growth hormone (GH) deficiency only came in 1966.

15 Short stature associated with GH deficiency has been estimated to occur with an incidence of between 1/4000 and 1/10000 live births. Most of these cases are both sporadic and idiopathic, but between 5 and 30% have an affected first-degree relative consistent with a genetic aetiology for the condition. Confirmation of the genetic aetiology of GH deficiency came from the molecular genetic analysis of familial short  
20 stature and the early demonstration of mutational lesions in the pituitary-expressed growth hormone (*GHI*) genes of affected individuals. Familial short stature may also be caused by mutation in a number of other genes (*eg POU1F1*, *PROPI* and *GHRHR*) and it is important to distinguish these different forms of the condition.

25 Growth hormone (GH) is a multifunctional hormone that promotes post-natal growth of skeletal and soft tissues through a variety of effects. Controversy remains as to the relative contribution of direct and indirect actions of GH. On one hand, the direct effects of GH have been demonstrated in a variety of tissues and organs, and GH receptors have been documented in a number of cell types. On the other hand, a  
30 substantial amount of data indicates that a major portion of the effects of GH are mediated through the actions of GH-dependent insulin-like growth factor I (IGF-I). IGF-1 is produced in many tissues, primarily the liver, and acts through its own

receptor to enhance the proliferation and maturation of many tissues, including bone, cartilage, and skeletal muscle. In addition to promoting growth of tissues, GH has also been shown to exert a variety of other biological effects, including lactogenic, diabetogenic, lipolytic and protein anabolic effects, as well as sodium and water retention.

Adequate amounts of GH are needed throughout childhood to maintain normal growth. Newborns with GH deficiency are usually of normal length and weight. Some may have a micropenis or fasting hypoglycemia in addition to their low linear growth, which becomes progressively retarded with age. In those with isolated growth hormone deficiency (IGHD), skeletal maturation is usually delayed in association with their height retardation. Truncal obesity, facial appearance younger than expected for their chronological age, high-pitched voice and delayed secondary dentition are often present. Skin changes similar to those seen in premature ageing may be seen in affected adults.

Familial IGHD comprises several different disorders with characteristic modes of inheritance. Those forms of IGHD known to be associated with defects at the *GHI* gene locus are shown in Table 1 together with the different types of underlying lesion so far detected.

**Table 1 Classification of inherited disorders involving the *GHI* gene**

Disorder	Mode of inheritance	Types of gene lesion responsible	GH protein	Deficiency state
IGHD IA	Autosomal recessive	Gross deletions, micro-deletions, nonsense mutations	Absent	Severe short stature. Anti-GH antibodies often produced upon GH treatment.
IGHD IB	Autosomal recessive	Splice site mutations	Deficient	Short stature. Patients usually respond well to exogenous GH
IGHD II	Autosomal dominant	Splice site and intronic mutations, missense mutations	Deficient	Short stature. Patients usually respond well to exogenous GH

The characterisation of these lesions has helped to provide explanations for the differences in clinical severity, mode of inheritance and propensity to antibody formation in response to exogenously administered GH, between these forms of IGHD. Most cases are sporadic and are assumed to arise from cerebral insults or defects that include cerebral oedema, chromosomal anomalies, histiocytosis, infections, radiation, septo-optic dysplasia, trauma, or tumours affecting the hypothalamus or pituitary. Magnetic resonance imaging examinations detect hypothalamic or pituitary anomalies in about 12% of patients who have IGHD.

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Although short stature, delayed growth velocity, and delayed skeletal maturation are all seen with GH deficiency, none of these is specific for this disorder; other systemic diseases may result in such symptoms. Provocative tests of GH deficiency include post-exercise, L-Dopa, insulin stress tolerance (IST), arginine, insulin-arginine, clonidine, glucagon, and propranolol. Inadequate GH peak responses (usually <7-10 ng/mL) differ from test to test. Testing for concomitant deficiencies of LH, FSH, TSH and ACTH should be performed to obtain a more accurate diagnosis and to plan optimal treatment.

15

Recombinant-derived GH is available worldwide and is administered by subcutaneous injection. To obtain an optimal outcome, children with IGHD are usually started on replacement therapy as soon as their diagnosis is established. The initial dosage of recombinant GH is based on body weight, but the exact amount used and the frequency of administration may vary between different protocols. The dosage increases with increasing body weight to a maximum during puberty and is usually discontinued by approximately 17 years of age.

20

Conditions that are treated with GH include (i) those in which it has proven efficacy and (ii) a variety of others in which its use has been reported but not accepted as standard practice. Disorders in which GH treatment has proven efficacy include GH deficiency, either isolated or in association with combined pituitary hormone deficiency (CPHD) and Turner syndrome. The clinical responses of individuals with

25

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the first two disorders to GH replacement therapy varies depending on (i) the severity of the condition and the age at which treatment is begun, (ii) recognition and response to treatment of associated deficiencies such as thyroid hormone deficiency, and (iii) whether treatment is complicated by the development of anti-GH antibodies. The outcome of treatment for individuals with Turner syndrome subjects varies with the severity of their short stature, their chromosomal complement, and the age at which treatment was begun.

Additional disorders in which the use of GH has been reported but for which its efficacy is not accepted as standard practice include treatment of certain skeletal dysplasias such as achondroplasia, Prader-Willi syndrome, growth suppression secondary to exogenous steroids in conditions such as the chronic autoimmune diseases, chronic renal failure, extreme idiopathic short stature, Russell-Silver syndrome, and intrauterine growth retardation. There are, in general, insufficient data to establish the efficacy of GH replacement therapy in treating these disorders owing to the limited number of subjects and the lack of use of standardized protocols.

The characterisation of familial IGHD at the molecular genetic level is important for several reasons. The identity of the locus involved will indicate not only the likely severity of growth retardation but, more importantly, the appropriateness or otherwise of the various therapeutic regimens now available. Further, detection of the underlying gene lesions serves to confirm the genetic aetiology of the condition. It may also have prognostic value in predicting (i) the severity of growth retardation and (ii) the likelihood of anti-GH antibody formation subsequent to GH treatment. In some instances, knowledge of the pathological lesion(s) can also help to explain an unusual mode of inheritance of the disorder and is therefore essential for the counselling of affected families. Finally, the characterisation of the mutational lesions responsible for cases of IGHD manifesting a dysfunctional (as opposed to a non-functional) GH molecule could yield new insights into GH structure and function.

At the cellular level, a single GH molecule binds two GH receptor molecules (GHR) causing them to dimerise. Dimerisation of the two GH-bound GHR molecules is

believed to be necessary for signal transduction, which is associated with the tyrosine kinase JAK-2. It has been suggested that the diverse effects of GH may be mediated by a single type of GHR molecule that can possess different cytoplasmic domains or phosphorylation sites in different tissues. When activated by JAK-2, these differing  
5 cytoplasmic domains can lead to distinct phosphorylation pathways, one for growth effects and others for various metabolic effects.

GH is a 22 kDa protein secreted by the somatotroph cells of the anterior pituitary. X-ray crystallographic studies have shown GH to comprise a core of two pairs of parallel  
10 alpha helices arranged in an up-up-down-down fashion. This structure is stabilised by two intra-molecular disulphide linkages (Cys53-Cys165 and Cys182-Cys 189). Two growth hormone receptor (GHR) molecules bind to two structurally distinct sites on the GH molecule, a process which proceeds sequentially by GHR binding first at site 1 and then at site 2. The binding of GHR to GH potentiates dimerisation of the GHR  
15 molecules.

Scanning mutagenesis studies of the GH molecule have yielded a picture of the binding interactions between GH and its receptor whilst site-directed mutagenesis has been used to probe the function of specific residues. Thus, substitution of Gly120 (in  
20 the third alpha helix of human GH) by Arg results in the loss of GHR binding to site 2 thereby blocking GHR dimerisation. Similarly, residue Phe44 of the human GH protein is important for binding the prolactin receptor. Finally, residues Asp115, Gly119, Ala122 and Leu123 have been shown to be critical for the growth enhancing potential of the murine GH molecule.

25 Interaction of the dimerised GHR with the intracellular tyrosine protein kinase JAK2 leads to tyrosine phosphorylation of downstream signal transduction molecules, stimulation of mitogen-activated protein (MAP) kinases and induction of signal transducers and activators of transcription (STAT proteins). In this way, GH is able to  
30 influence the expression of multiple genes through a number of different signalling pathways.

Several different GH isoforms are generated from expression of the *GHI* gene (*GHI* reference sequence is shown in Figure 5). In 9% of *GHI* transcripts, exon 2 is spliced to an alternative acceptor splice site 45bp into exon 3, thereby deleting amino acid residues 32 to 46 and generating a 20 kDa isoform instead of the normal 22 kDa protein. This 20 kDa isoform appears to be capable of stimulating growth and differentiation. The factors involved in determining alternative acceptor splice site selection are not yet characterised but are clearly of a complex nature. A 17.5 kDa isoform, resulting from the absence of codons 32 to 71 encoded by exon 3, has also been detected in trace amounts in pituitary tumour tissue. Splicing products lacking either exons 3 and 4 or exons 2, 3 and 4 have been reported in pituitary tissue but these appear to encode inactive protein products. A 24 kDa glycosylated variant of GH has also been described. The amino acid sequence of the major 22 kDa isoform is presented in Figure 6, which shows the nucleotide sequence of the *GHI* gene coding region and amino acid sequence of the protein including the 26 amino acid leader peptide. Lateral numbers refer to amino acid residue numbering. Numbers in bold flanking vertical arrows specify the exon boundaries. The termination codon is marked with an asterisk.

The gene encoding pituitary growth hormone (*GHI*) is located on chromosome 17q23 within a cluster of five related genes (Figure 1). This 66.5 kb cluster has now been sequenced in its entirety [Chen *et al. Genomics* 4: 479-497 (1989) and see Figure 5]. The other loci present in the growth hormone gene cluster are two chorionic somatomammotropin genes (*CSH1* and *CSH2*), a chorionic somatomammotropin pseudogene (*CSHP1*) and a growth hormone gene (*GH2*). These genes are separated by intergenic regions of 6 to 13 kb in length, lie in the same transcriptional orientation, are placentally expressed and are under the control of a downstream tissue-specific enhancer. The *GH2* locus encodes a protein that differs from the *GHI*-derived growth hormone at 13 amino acid residues. All five genes share a very similar structure with five exons interrupted at identical positions by short introns, 260bp, 209bp, 92bp and 253bp in length in the case of *GHI* (Figure 2).

Exon 1 of the *GH1* gene contains 60bp of 5' untranslated sequence (although an alternative transcriptional initiation site is present at -54), codons -26 to -24 and the first nucleotide of codon -23 corresponding to the start of the 26 amino acid leader sequence. Exon 2 encodes the rest of the leader peptide and the first 31 amino acids of mature GH. Exons 3-5 encode amino acids 32-71, 72-126 and 127-191, respectively. Exon 5 also encodes 112bp 3' untranslated sequence culminating in the polyadenylation site. An *Alu* repetitive sequence element is present 100bp 3' to the *GH1* polyadenylation site. Although the five related genes are highly homologous throughout their 5' flanking and coding regions, they diverge in their 3' flanking regions.

The *GH1* and *GH2* genes differ with respect to their mRNA splicing patterns. As noted above, in 9% of *GH1* transcripts, exon 2 is spliced to an alternative acceptor splice site 45bp into exon 3 to generate a 20 kDa isoform instead of the normal 22 kDa. The *GH2* gene is not alternatively spliced in this fashion. A third 17.5 kDa variant, which lacks the 40 amino acids encoded by exon 3 of *GH1*, has also been reported.

The *CSH1* and *CSH2* loci encode proteins of identical sequence and are 93% homologous to the *GH1* sequence at the DNA level. By comparison with the *CSH* gene sequences, the *CSHP1* pseudogene contains 25 nucleotide substitutions within its "exons" plus a G→A transition in the obligate +1 position of the donor splice site of intron 2 that partially inactivates its expression.

A number of biallelic restriction fragment length polymorphisms (RFLPs) have been reported within the GH gene region. Five of these (two *Bgl*II, two *Msp*I, one *Hinc*I) occur in Caucasians and blacks whereas a further *Bam*HI polymorphism occurs predominantly in blacks. Strong linkage disequilibrium has been observed between these polymorphisms consistent with the relatively recent evolutionary origin of the gene cluster. The *Hinc*II and *Bam*HI polymorphisms occur immediately 5' to the *GH1* gene. An *Rsa*I polymorphism occurs in the *GH1* promoter region resulting from an A/G dimorphism at nucleotide -75 whilst a relatively frequent *Sph*I polymorphism

remains to be fully characterised. A highly informative (83% heterozygosity) variable number repeat polymorphism has been located some 19kb 3' to the *GHI* gene; formatted for PCR, the 18 distinct alleles of this polymorphism can be distinguished by fragment size (201 to 253bp).

5

Finally, the *GHI* gene promoter region has been found to exhibit a very high level of sequence polymorphism with 8 variant nucleotides within a 134 bp stretch: -75 (A or G), -57 (G or T), -6 (A or G), -1 (A or T or C), +3 (G or C), +16 (A or G), +25 (A or C), +59 (T or C) [Giordano *et al. Human Genetics* 100: 249-255 (1997)]. (Figure 3).  
10 These eight variable positions can be ascribed to 12 different haplotypes ranging in frequency from 2% to 31% in the general population. The polymorphisms at positions -1, +3 and +59 are predicted to cause amino acid substitutions in the *GHD* protein, putatively encoded by this region of the *GHI* gene promoter (see below). The 8 sequence variants occur in the same positions in which the *GHI* gene differs from the  
15 other placentally expressed genes suggesting that the mechanism might be gene conversion and that the placental genes have served as donors of the converted sequences.

Since the first *GHI* gene deletions were reported, a variety of more subtle lesions have  
20 been described. In some cases, these lesions have been associated with unusual types of GH deficiency and are potentially important as a means of obtaining new insights into GH structure and function

The gene encoding growth hormone (*GHI*) was one of the first human genes to be  
25 cloned and the first gross gene deletions (6.7kb type) responsible for inherited growth hormone deficiency were soon detected by Southern blotting.

**Table 2 Gross deletions involving the *GHI* gene, or in the vicinity of the *GHI* gene, that cause GH deficiency and short stature**

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Deletion size (kb)	Loci involved	Comments	Post-treatment antibodies

			present?
6.7	<i>GH1</i>	Swiss family	yes
6.7	<i>GH1</i>	Japanese family	yes
6.7	<i>GH1</i>	Argentinan family of Spanish ancestry. Homozygous.	yes
6.7	<i>GH1</i>	Austrian family	yes
6.7	<i>GHI</i>	Brazilian family	yes
6.7	<i>GH1</i>	Patient with short stature and cystic fibrosis	yes
6.7	<i>GH1</i>	Various	no
7.6	<i>GHI</i>	Iraqi, Yemeni and Iranian families	no
7.6	<i>GH1</i>	Italian family. Homozygous. Consanguinous marriage	yes
7.6	<i>GHI</i>	Italian and Turkish families	yes
7.6	<i>GH1</i>	Spanish family	no
7.6	<i>GH1</i>	Various	yes
7.0	<i>GH1</i>	Canadian family	yes
7.0	<i>GH1</i>	Mexican family	yes
7.0	<i>GH1</i>	Chinese family. Homozygous	no - No treatment with GH.
45	<i>GH1, CSHP1, CSH1, GH2</i>	Turkish family. Homozygous. Consanguinous marriage	yes
45	<i>GH1, CSHP1, CSH1, GH2</i>	Italian family. Homozygous	yes
45	<i>GH1, CSHP1, CSH1, GH2</i>	Italian family. Homozygous. Consanguinous marriage	yes
45	<i>GH1, CSHP1, CSH1, GH2</i>	"Asian" family	no

?	<i>CSH1, GH2, CSH2</i>	Italian family. Heterozygous	no
?	<i>CSH1, GH2, CSH2</i>	Danish family. Compound heterozygous for non-identical deletions	no
Double	(i) <i>GHI</i> (6.7kb)  (ii) <i>CSH1, GH2, CSH2</i> (~32kb)	French origin (Romany). Homozygous. Consanguinous marriage.	yes

All gross deletions involving the *GHI* gene result in severe (type IA) deficiency, characterised by the total absence of GH. ~70% of characterised deletions of the *GHI* gene are 6.7 kb in length, whilst most of the remainder are of 7.6 kb or 7.0 kb (Table 2). In addition, several examples of much more infrequent deletions have been reported.

In recent years, various attempts have been made to move away from Southern blotting toward PCR-based approaches as a mutation screening tool. Homozygous *GHI* gene deletions have been fairly readily detected by PCR amplification of the *GHI* gene and flanking regions followed by restriction enzyme digestion of the resulting PCR products. Although this approach has been used successfully to exclude homozygosity for a *GHI* gene deletion in at-risk pregnancies, it is however unable to distinguish homozygosity for the wild-type gene from heterozygosity for a gene deletion. It would also fail to detect deletions other than the relatively short 6.7, 7.0 and 7.6kb deletions that remove only the *GHI* gene.

PCR primers have been designed which immediately flank the *GHI* gene and which generate a 790bp fragment from control DNA samples. Absence of this fragment was held to be indicative of a *GHI* gene deletion but the use of “non-specific PCR fragments” as internal controls for PCR amplification must make the reliability of this method somewhat suspect.

As well as gross deletions, three micro-deletions of the *GHI* gene have been reported; two of these patients were also heterozygous for the 6.7 kb *GHI* gene deletion (Table 3).

- 5 Only six different single base-pair substitutions have been reported from within the coding region of the *GHI* gene (Table 4). Two of these are nonsense mutations converting amino acid residues Trp-7 and Glu-4 in the signal peptide to stop codons. These mutations are the only known *GHI* gene lesions to cause type IA deficiency that are not gene deletions. Since these lesions predict termination of translation within the
- 10 signal peptide, they would be incompatible with the production of a functional GH molecule. The other four single base-pair substitutions are missense mutations that result in the production of dysfunctional growth hormone molecules.

15 Table 3 Micro-deletions in the *GHI* gene causing GH deficiency and short stature

Deficiency type	Deletion (Lower case letters denote the deleted bases. ^ specifies the location of the numbered codon immediately downstream.)	Codon (Numbering is relative to translational initiation codon ATG at -26.)	Post-treatment antibodies present?
IA	GCCTG^CTCTGcCTGCCCTGGC	-11	yes
II	CCCCAGGCGGggatgggggagacctgtaGTC AGAGCCC	Intron 3 (del+28 to +45)	no
IA	TCTGT^TTCTCagAGTCTATTCC	54	no

Table 4 Single base-pair substitutions in the *GHI* coding region causing GH deficiency and short stature

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Deficiency type	Nucleotide substitution	Amino acid substitution	Codon (numbering relative to	Post-treatment antibodies
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			translational initiation codon ATG at -26)	present?
IA	ACA→GCA	Thr→Ala	-24	no
IA	TGG→TAG	Trp→Term	-7	no
IA	GAG→TAG	Glu→Term	-4	yes
II	CGC→TGC	Arg→Cys	77	no
?	GAC→GGC	Asp→Gly	112	no
?	CGC→CAC	Arg→His	183	no

Single base-pair substitutions in the promoter region were first sought by sequencing the promoter region of the *GHI* gene (between -60 and +70 relative to the transcriptional initiation site) in three Chinese patients with IGHD IA and 2 controls.

- 5 Several differences were noted but these were probable polymorphisms and were not characterised further. As mentioned above, the promoter region of the *GHI* gene has subsequently been shown to exhibit a very high level of sequence polymorphism with 8 variant nucleotides within a 134 bp stretch (Figure 3). However, they were not found to be over-represented in patients as compared to controls.

10

- GHI* promoter variation has also been separately investigated and a total of 22 variant polymorphic sites were detected, mostly single base-pair substitutions: 17 of these occurred in a 550 bp region 5' to the ATG initiation codon, three occurred around position -1075 5' to ATG, and two occurred within intron 1 (IVS1) at positions 76 and 15 219 respectively. All except four of these variants were also noted in controls but these four variants were not considered to be the cause of the growth hormone deficiency. Only one of the variant sites occurred within a sequence homologous to a transcription factor binding site: the alternative presence of CCAGA and GAGAG sequences at -333 within a potential (but not proven) NF-1 binding site.

20

Therefore, to date, no mutations of pathological significance have been reported in the *GHI* gene promoter.

Single base-pair substitutions affecting mRNA splicing have also been described in the *GH1* gene. Most are associated with a comparatively rare dominant form of GH deficiency (Table 5).

- 5 The transversions in the intron 4 donor splice site have been shown by mRNA *in vitro* expression analysis of transfected cells to activate a cryptic splice site within exon 4, 73bp 5' to the exon 4 donor splice site. This would predict the generation of an aberrantly spliced product lacking amino acids 103-126 encoded by exon 4 and, as a consequence of a shift in the reading frame, the incorporation of 94 novel amino acids
- 10 including 29 resulting from read-through of the normally untranslated 3' non-coding region of the *GH1* gene.

**Table 5 Single base-pair substitutions affecting mRNA splicing and causing GH deficiency and short stature**

Deficiency type	Nucleotide substitution/ position	Splice site	Ethno-geographic origin/zygosity
II	G→A, +1	IVS3 donor	Sweden, North America, Northern Europe, South Africa, Chile/heterozygous
II	G→C, +1	IVS3 donor	Turkish/ heterozygous
II	G→A, +5	IVS3 donor	Chilean/ heterozygous
II	G→C, +5	IVS3 donor	?
II	T→C, +6	IVS3 donor	Turkish/ heterozygous Asian/ heterozygous
II	G→A, +28	IVS3 donor	?/heterozygous
IB	G→C, +1	IVS4 donor	Saudi Arabian/ homozygous
IB	G→T, +1	IVS4 donor	Saudi Arabian/ homozygous
IB	G→C, +5	IVS4 donor	?

15

Since the region of the GH protein encoded by exons 4 and 5 is thought to be important for correct targeting of the protein to secretory granules, it has been

predicted that this aberrant protein would not be secreted normally. However, no antibodies to exogenous GH have been noted in patients with type IB GH deficiency. The avoidance of immune intolerance may thus indicate that at least some of the aberrant protein product could be secreted and that it could be partially stable in the circulation. By contrast, the five known splicing mutations within IVS3 (Table 5) are associated with a type II deficiency state manifesting autosomal dominant inheritance through the affected families.

GH deficiency patients with truncating *GHI* mutations or homozygous gene deletions  
10 are at considerable risk of developing anti-GH antibodies upon GH treatment. By  
contrast, we are not aware of any reports describing allo-antibody formation in patients  
with either missense mutations or single base-pair substitutions within splice sites.

Until now, no other correlations between mutant genotype and clinical phenotype have been reported. The requisite data in the published literature are sparse and very variable in quality, but we have attempted a crude meta-analysis as a means of gauging whether or not patients with gross gene deletions differ from patients with splice site mutations in terms of their clinical and phenotypic sequelae. The height of the patients with *GHI* deletions was found to be on average 7.3 SD below the age-adjusted mean (n=29), as compared with an average of 5.4 SD below the mean (n=17) for the patients with *GHI* splicing mutations. Although bone age delay was greater and growth velocity lower in the deletion patients, such findings are very difficult to interpret since they may be subject to bias of ascertainment.

Since most cases of familial GH deficiency hitherto described are inherited as an autosomal recessive trait, some examples of the inherited deficiency state are likely to have gone unrecognized owing to small family size. Similarly, cases of GH deficiency resulting from *de novo* mutations of the *GHI* gene could be classified as sporadic, and a genetic explanation for the disorder would neither be entertained nor sought. Finally, depending upon the stringency of the criteria used for defining the deficiency state, it may be that examples of milder forms of GH deficiency may never have come to clinical attention. For these reasons, current estimates of the prevalence of GH

deficiency could be inaccurate and may therefore seriously underestimate the true prevalence in the population.

The strict adherence to formal definitions of what constitutes GH deficiency (as mentioned above, severe IGHD is usually defined as  $<-4.5$  SD in height) and the fairly uniform acceptance of conservative criteria in selecting patients for further study [Shalet SM *et al. Endocrine Rev.* 19: 203-223 (1998)] would have served to ensure that the described *GHI* mutational spectrum was not only far from complete but also unrepresentative of the wider mutational spectrum. Thus, mutations responsible for milder forms of GH deficiency (eg missense mutations within the coding region of the gene or promoter mutations) would have been much less likely to come to clinical attention. Indeed, this may go some way toward explaining why only four different missense mutations have so far been reported in the *GHI* gene, a finding which is virtually unprecedented for a fairly prevalent disorder that has been studied at the molecular level for nearly 20 years.

The absence of GH produces a readily recognisable and severe clinical phenotype that has been extensively studied. In those reported studies of less severely affected patients in which patient selection criteria have actually been identified, patient ascertainment strategies have generally used the deviation of an individual's height from the mean height for their age as a diagnostic indicator of growth failure.

We have proposed that the relaxation of the criteria applied in selecting patients for study would be likely to lead to the inclusion of patients manifesting milder forms of GH deficiency, which could therefore yield a novel set of underlying mutational lesions. Some of these milder lesions could give rise to stable, yet dysfunctional, GH molecules that would exhibit normal immunological reactivity but little or no biological activity. On the basis of radio-immunoassay test results, dysfunctional GH molecules would have been erroneously regarded as normal. If such dysfunctional variants were to turn out to be common, then it would follow that GH deficiency is being under-diagnosed as a result of our current dependence on radio-immunoassay-

based GH "function tests". Further, it would demonstrate an urgent need for the development of a true functional diagnostic assay.

We believe that growth velocity (GV) is a more sensitive indicator of growth failure than absolute height measurements. The use of GV in conjunction with an assessment of bone age delay (also failure due to GH deficiency); and other parameters being normal, together with a degree of clinical concern, has allowed us to identify a group of patients unified by their phenotype, who are less severely affected than patients with no GH, but who are more likely to have lesions of the *GHI* gene than those selected on the basis of height measurements alone.

Accordingly, the present invention provides a detection method for detecting a variation in *GHI* effective to act as an indicator of GH dysfunction in a patient, which detection method comprises the steps of:

- (a) obtaining a test sample comprising a nucleotide sequence of the human *GHI* gene from the patient; and
- (b) comparing the sequence obtained from the test sample with the standard sequence known to be that of the human *GHI* gene, wherein a difference between the test sample sequence and the standard sequence indicates the presence of a variation effective to act as an indicator of GH dysfunction (hereinafter "GH variant") characterised in that the test sample is obtained from a patient exhibiting the following cumulative criteria:

- (i) growth velocity below the 25<sup>th</sup> centile for age;
- (ii) bone age delay of at least two years according to the Tanner-Whitehouse scale; and
- (iii) no other disorder known to cause inclusion in criteria (i) to (ii) above.

The present invention therefore further provides a GH variant detected by or detectable according to the above-described method of this invention.

With respect to the cumulative criteria (i) to (iii), each criterion may be assessed according to known methods and parameters readily available and described in the art, as elaborated further below:

- 5 (i) Tanner JM, Whitehouse RH. *Atlas of Children's Growth*. London: Academic Press, 1982; and Butler *et al. Ann. Hum. Biol.* 1990; 17: 177-198 (1990) are sources for statistics enabling a determination of the first criterion, viz that the growth velocity of the patient is less than the 25<sup>th</sup> centile for the patient's age.
- 10 (ii) The Tanner-Whitehouse scale for assessing years of bone age delay is described by Tanner JM, Whitehouse RH, Cameron N *et al* in *Assessment of Skeletal Maturity and Prediction of Adult Height*. London: Academic Press, 1983.
- (iii) Since short stature may also be secondary to conditions other than GH  
15 dysfunction, test samples from patients suffering from such disorders are excluded from the method of the invention. That the patient is suffering from no other disorder that might give rise to similar symptoms to that of GH dysfunction is determined by baseline investigations. "Baseline investigations" therefore include tests to exclude, particularly, hypothyroidism; malabsorption syndromes *eg* coeliac disease; renal and  
20 hepatic diseases; haematological disorders, such as anaemia; and a karyotype to check that a chromosome disorder such as Turner syndrome is not the cause of the growth failure. The patient may also have had a thorough clinical examination in order to exclude other causes of growth failure, for example, cardiac disease including congenital heart disease; chronic auto-immune conditions, such as rheumatoid arthritis  
25 and inflammatory bowel disease; chronic respiratory conditions, such as severe asthma or cystic fibrosis; and skeletal problems, such as achondroplasia. A full medical history will also have been taken and used to complement the medical examination in order to aid the exclusion not only of the physical disorders identified above but also of psycho-social deprivation, another well-recognised cause of growth  
30 failure in childhood.

Optionally, the patient may also have been subjected to one or more growth hormone function tests. The term "growth hormone function tests" refers to tests of growth hormone secretion, such as those provocative tests mentioned hereinbefore, particularly the insulin stress test (IST).

5

GH function tests are usually carried out on patients who are short; have been assessed and had their height monitored over more than one visit to the endocrine clinic; have no other detectable cause for their growth failure; and therefore warrant being subjected to an assessment of their ability to produce growth hormone secretion from their pituitary gland following an appropriate stimulus, such as the profound drop in blood sugar that results from the administration of intravenous insulin.

10

Preferably, in the selection criteria for use in the detection method of this invention, a further criterion may also be applied in addition to (i) to (iii) above, namely:

15

(iv) growth failure, defined as a growth pattern [delineated by a series of height measurements; Brook CDG, Ed., *Clinical Paediatric Endocrinology*, 3rd Ed., (1996) Blackwell Science. Chapter 9, p141] which, when plotted on a standard height chart [Tanner *et al. Arch. Dis. Child* 45: 755-762 (1970)], indicates a predicted adult height outside the patient's predicted target adult height range.

20

Also useful as a reference is Tanner and Whitehouse. *Arch. Dis. Child* 51: 170-179 (1976)]. A patient's target adult height range is calculated as the mid-parental height (MPH) with the range being the 10th to 90th centile for MPH, which is sex-dependent:

25

MPH if male = [father's height + (mother's height + 13)]/2 + or - in the range of from 6 to 8cm, usually 7.5cm; and

MPH if female = [(father's height - 13) + mother's height]/2 + or - in the range of from 6 to 8 cm, usually 6cm

30

These are standard tests and measurements used in the field and any other acceptable method of calculation can be used to determine growth failure, although the above-described method based on the description in Brook (*ibid*, 1996) regarding the formula to apply for predicting the limits of the target height range and on the description in Tanner (*ibid*, 1970) regarding the standard height charts are preferred according to this invention.

In the detection method according to this invention, therefore, although current height may be measured in order to apply the above-noted criteria, this is not in itself a criterion used for selection of patients in this method. As mentioned above, prior art methods rely on standard deviation from 'normal' height (*ie* absolute growth) as the criterion for selecting patients. The present invention does not require inclusion of such criterion and therefore the present invention provides a detection method in which absolute height is or may be excluded as a selection criterion.

Increasing the breadth of the *GHI* mutational spectrum will inevitably lead to a redefinition of inherited GH deficiency in molecular genetic terms. Furthermore, the recognition of novel types of short stature must eventually require the reclassification of GH deficiency as a disease entity. This will obviously have important implications for the screening and identification of individuals with short stature in whom the use of growth hormone treatment might be beneficial.

The test sample obtained from the patient in the detection method of the invention preferably comprises genomic DNA extracted from patient lymphocytes by standard procedures. *GHI* gene analysis is thereafter preferably carried out according to the following steps:

1(a). PCR amplification of a 3.2 kb fragment containing the *GHI* gene in its entirety (promoter, five exons of the coding region, introns and untranslated regions) followed by the nested PCR of smaller, overlapping constituent fragments using primers designed so as to ensure *GHI* gene specificity. As well as using six known primers, the design of novel *GHI*-specific primers was found to be essential in order to avoid



cross-contamination emanating from inadvertent PCR amplification of the paralogous, closely linked and highly homologous *GH2*, *CSH1* and *CSH2* genes, and the *CSHP1* pseudo-gene; and/or

5 1(b). PCR amplification of the DNA sequence spanning the Locus Control Region (LCR), an enhancer region that affects the level and time of *GHT* transcription. The LCR is located ~14 kb 5' to the *GHI* gene and is responsible for the coordinate expression of the genes in the GH gene cluster. PCR amplification was carried out, using novel oligonucleotide primers, on two overlapping fragments (254 bp and 258  
10 bp) in all patients;

2. Mutational screening of the *GHI* gene fragments by Denaturing High Performance Liquid Chromatography (DHPLC) using the Transgenomic WAVE™ System [O'Donovan *et al. Genomics* 52: 44-49 (1998)]. This screening method was selected  
15 for use since it is extremely rapid, cheap, sensitive and reproducible and exhibits, at least in our hands, a detection efficiency >95%. "Bandshifts" detected by DHPLC would represent potential DNA sequence variants; and

3. Characterisation of any such variants by DNA sequencing (either by automated or  
20 manual methods); and, optionally, but preferably also

4. Functional characterisation of *GHI* gene lesions using methodology appropriate to the location of the lesion and the inferred mechanism of dysfunction.

25 Therefore, the present invention further provides novel *GHI*-specific primers for use in the analysis of *GHI* as described above and in the examples, which primers include:

novel primers suitable for use in the DHPLC step (see Example 3, Table 6, for further details):

30

CTC CGC GTT CAG GTT GGC (GHD1F);

AGG TGA GCT GTC CAC AGG (GHD1R);

- CTT CCA GGG ACC AGG AGC (GHD2R);  
CAT GTA AGC CAA GTA TTT GGC C (GHD3F);  
GGA GAA GGC ATC CAC TCA CGG (GHD4R);  
TCA GAG TCT ATT CCG ACA CCC (GHD5F);  
5 CGT AGT TCT TGA GTA GTG CGT CAT CG (GHD6R); and  
TTC AAG CAG ACC TAC AGC AAG TTC G (GHD7F);

and primers suitable for use in the LCR step (all 5'→3', see also Example 5):

- 10 GTGCCCCAAGCCTTTCCC (LCR15: 1159-1177);  
TGTCAGATGTTTCAGTTCATGG (LCR13: 1391-1412);  
CCTCAAGCTGACCTCAGG (LCR25: 1346-1363); and  
GATCTTGGCCTAGGCCTCG (LCR23: 1584-1602).

- 15 The detection method of the invention and the GH variant identifiable or detectable  
thereby can give rise to the following additional advantages:

1. Expansion of the known spectrum of *GHI* gene mutations by identification and characterisation of new lesions.
- 20 2. Evaluation of the role of *GHI* gene mutations in the aetiology of short stature.
3. Identification of the mode of inheritance of novel *GHI* gene lesions.
4. Elucidation of the relationship between mutant genotype and clinical phenotype.  
This is deemed essential for the early detection and appropriate clinical management of GH deficiency.
- 25 5. Evaluation of the effects of *GHI* mutations on the structure and function of the GH molecule. This is particularly important for the assessment of those children with a clinical phenotype at the milder end of the clinical spectrum of short stature. In this group of patients, dysfunctional GH may be produced that is immunologically active and therefore falls within the normal range in GH function tests.
- 30 6. Development of rapid DNA diagnostic tests for inherited GH deficiency
7. Assessment of our postulate that GH deficiency is currently underdiagnosed and underestimated in the population.

Therefore, the characterisation of further, naturally occurring *GHI* lesions promises to be of considerable importance to studies of GH structure, function and expression. Studies of novel coding sequence variants should increase our understanding not only of GH function, but also of the interactions between GH and its receptor (GHR), and the process of GHR-mediated signal transduction. Insights obtained could be relevant to the rational design of a new generation of therapeutic agents. Similarly, studies of naturally-occurring *GHI* lesions in the promoter region should provide new insights into the control of *GHI* gene expression. Thus it may be seen that a broad spectrum of mutational lesions will necessarily improve our understanding of the relationship between mutant genotype and clinical phenotype in inherited forms of GH deficiency. Clearly, these studies are essential for the early detection and appropriate clinical management of familial GH deficiency.

The present invention therefore further provides a variant of *GHI*, which differs from *GHI* and is detectable by the method according to the invention but is not detectable by methods used hitherto, such as those reliant on patient selection criteria based primarily on height. Such *GHI* variants of the invention include those characterised in Example 6 and Table 7 hereinafter.

As indicated hereinbefore, current tests to assess GH secretion are many and varied and no single currently available investigation is ideal. Since the secretion of human GH is pulsatile, and because the amplitude and frequency of the GH pulses are extremely variable (being influenced by multiple internal and external factors including sleep, exercise, stress and the pubertal stage of the individual concerned), those tests that yield the best information require close supervision of the patient in a dedicated investigation ward. The tests are therefore time-consuming, expensive, and cause considerable stress and distress to the patient and their family. The insulin stress test (IST) is of particular note; it is used by many doctors, as mentioned above, to assess GH secretion but deaths have occurred owing to the hypoglycaemia induced in the patient as a necessary requirement of its successful implementation. It is therefore of paramount importance that the decision to perform an investigation, such as an IST,

is most carefully considered before it is given a place in the assessment of a short child. The development of a DNA test for use in screening short patients would therefore have many advantages over the other tests currently available.

5 Accordingly, the present invention provides a screening method for screening a patient suspected of having dysfunctional GH, which screening method comprises the steps of:

- 10 (a) obtaining a test sample comprising a nucleotide sequence of the human *GHI* gene from the patient; and
- (b) comparing a region of the sequence obtained from the test sample with the corresponding region of a predetermined sequence characterised in that the predetermined sequence is selected from a GH variant detectable according to the above-described method of the present invention.

15 More specifically, the screening method of the invention is characterised in that the predetermined sequence is an oligonucleotide having a nucleic acid sequence corresponding to a region of a variant *GHI* gene, which region incorporates at least one variation when compared with the corresponding region of the wild type sequence.

20 Especially preferred is when the variation is one detectable by the detection method of the invention, such as any of those identified in Example 6 and Table 7 hereinafter. Preferably, the test sample comprises genomic DNA, which may be extracted by conventional methods.

25 Therefore, the present invention further provides a screening method for determining GH dysfunction, comprising:

- (a) obtaining a first human tissue suspected of GH dysfunction; and
- 30 (b) comparing the *GHI* gene or *GHI* transcript, or fragment therefrom (eg cDNA), in the first human tissue to the corresponding gene, transcript or fragment of a *GHI*

variant obtainable from a second human tissue derived from a human exhibiting the following cumulative criteria:

- (i) growth velocity below the 25<sup>th</sup> centile for age;
- 5 (ii) bone age delay of at least two years according to the Tanner-Whitehouse scale; and
- (iii) no other disorder known to cause inclusion in criteria (i) to (ii) above; and, optionally,
- (iv) growth failure defined as a growth pattern [delineated by a series of height measurements; Brook CDG, Ed., *Clinical Paediatric Endocrinology*, 3rd Ed., (1996)
- 10 Blackwell Science. Chapter 9, p141] which, when plotted on a standard height chart [Tanner *et al. Arch. Dis. Child* 45: 755-762 (1970)], indicates a predicted adult height outside the patient's predicted target adult height range.

In the screening method of the invention, the comparison step may be carried out in  
15 conventional manner, for example by sequencing the appropriate region of the *GHI* gene, particularly in the case where relatively few variants are to be detected/compared. Where relatively large numbers of variants are involved, DNA chip technology may be employed, wherein the chip is a miniature parallel analytical device that is used to screen simultaneously for multiple mutations by hybridisation of  
20 labelled sample DNA (cDNA or genomic DNA derived from the patient) to microarrays of mutation-specific oligonucleotide probes immobilised on a solid support [Southern, *Trends Genet.* 12: 110-115 (1996)].

The advantage of a DNA screening method according to the invention over current  
25 tests include:

1. It involves, for the patient, only a single blood test that can be performed in a clinic. Hospital admission, prolonged medical supervision and repeated blood sampling would not be required as is the case for the majority of currently-available tests. There  
30 would therefore be a reduction in the expense incurred, the use of specialist time and the distress caused for each patient tested.

2. Earlier diagnosis of GH deficiency in patients would become possible. The ease with which the DNA screen can be performed would allow the clinician to consider such an investigation much earlier in the management of a patient than might otherwise be the case. Currently, owing to the problems inherent in tests for GH secretion, doctors will assess children in the out-patient clinic over a long period of time, sometimes several years, before they will subject a child to an IST. The early diagnosis of a genetic aetiology for GH deficiency would enable earlier treatment with GH thereby bringing forward the opportunity to treat patients appropriately by months, or even years in individuals with a less severe phenotype.

10

3. More patients could be tested for GH dysfunction. The ease of the DNA test would allow the doctor to perform it as part of the initial assessment of all short patients at their first visit to the endocrine clinic. This is likely to reveal patients with lesions of the *GHI* gene that cause severe growth problems and also those with milder lesions (e.g. missense mutations in the coding region). These patients may not previously have come to clinical attention because their clinical/phenotypic problems would not have been severe enough to warrant an IST, but they might nevertheless still benefit from treatment with GH.

15

4. Early identification of patients who will require life-long treatment with GH would be possible. These patients could be identified and treated appropriately without recourse to either initial testing or re-testing for GH secretion, or the use of a period without GH to assess their progress (a "trial without treatment").

20

5. Easy and early identification of family members with GH dysfunction would become available. Once the genetic lesion responsible for growth problems has been identified in an individual, it is relatively easy to assess other family members for the same genetic lesion and to ascertain whether they would also gain benefit from treatment with GH.

25

30

6. Accuracy of diagnosis should increase. Tests for GH secretion are notorious for their variability in terms of reproducibility of assay results, both within and between

laboratories. DNA screening would make this problem a thing of the past. In addition, GH secretion test results can be very difficult to interpret in certain situations, for example, if the patient is also hypothyroid or if they have delayed puberty. DNA screening would remove this doubt and prevent delay in the initiation  
5 of GH treatment for those patients in whom its use would be beneficial

Accordingly, the present invention further provides a kit suitable for use in carrying out the screening method of the invention, which kit comprises:

- 10 (a) an oligonucleotide having a nucleic acid sequence corresponding to a region of a variant *GHI* gene, which region incorporates at least one variation from the corresponding wild-type sequence; and
- (b) an oligonucleotide having a nucleic acid sequence corresponding to the wild-type sequence in the region specified in (a); and, optionally,
- 15 (c) one or more reagents suitable for carrying out PCR for amplifying desired regions of the patient's DNA.

Such reagents may include, for example, PCR primers corresponding to the exon of the *GHI* gene, and/or primers mentioned herein, especially novel primers mentioned  
20 hereinabove; and/or other reagents for use in PCR, such as *Taq* DNA polymerase.

Preferably, the oligonucleotides in the kit comprise in the range of from 20 to 25 base-pairs, such as 20 base-pairs for the variant sequences and either 20 for the wild-type in the case where the variant is a single base-pair substitution or 25 base-pairs where the  
25 variant is a 5 base-pair deletion. In any case, the oligonucleotides must be selected so as to be unique for the region selected and not repeated elsewhere in the genome.

Obviously, in the situation where it is desired to screen for multiple variations, such as in the range of from 15 to 20 or more, this would necessitate a kit comprising up to 40  
30 oligonucleotides or more. In the alternative screening method, therefore, using DNA chip technology, the present invention provides a plurality of oligonucleotides as defined in kit component (a) above immobilised on a solid support.

*GHI* variants detectable by the detection method of this invention may have additional uses than as standards in a screening test for GH dysfunction. For example, variants other than those where the variation is in the promoter region of the *GHI* gene may be used to treat a patient wherein GH production is over-stimulated, such as in cases of  
5 pituitary gigantism or acromegaly.

Accordingly, the present invention further provides a composition comprising a variant of *GHI*, especially a variant detectable by the detection method of this invention and identified herein, in association with a pharmaceutically acceptable  
10 carrier therefor.

Furthermore, the invention provides:

- (a) a nucleic acid sequence encoding a variant of *GHI*
- 15 (b) a sequence substantially homologous to or that hybridises to sequence (a) under stringent conditions; or
- (c) a sequence substantially homologous to or that hybridises under stringent conditions to the sequence (a) or (b) but for the degeneracy of the genetic code; or
- (d) an oligonucleotide specific for any of the sequences (a), (b) or (c).

20

Also provided are:

- (a) a vector comprising the nucleic acid sequence described above;
- (b) a host cell comprising the vector (a), such as a bacterial host cell; and
- 25 (c) a process for preparing a variant of *GHI*, which process comprises:
  - (i) culturing the host cell (b); and
  - (ii) recovering from the culture medium the variant of *GHI* thereby produced.

The present invention will now be illustrated with reference to the following  
30 Examples.



## **Example 1 – Patient Selection**

### **Sources of Patients**

Children with short stature have been identified through referral to the Regional  
5 Paediatric Growth, Endocrine and Diabetes Service at the University of Wales College  
of Medicine in Cardiff and by collaboration with other similar UK centres (*viz.*  
Newport, Birmingham, Bristol, Wrexham, Liverpool, Stoke-on-Trent, Portsmouth and  
Southampton). A full clinical history has been taken including family history,  
pedigree, documentation of growth parameters and previously-performed endocrine  
10 investigations. Accurate auxology was recorded wherever possible for the index case,  
parents and siblings. Blood samples for molecular genetic analysis were taken from  
the index case and appropriate close relatives. Further families were referred by  
Professor John A. Phillips III (Nashville, TN, USA), Dr Mohamad Maghnie (Pavia,  
Italy) and Dr Tamas Niederland (Gyor, Hungary). To date, samples from 69 GH-  
15 deficient families have been collected. Criteria used for the selection of patient 1  
were:

- Growth velocity <25<sup>th</sup> centile
- Bone age delay 3.5-4 years
- 20 Growth well below lower limit of % target height range
- Growth hormone secretion tests normal
- All other investigations normal

### **25 Example 2 - Polymerase chain reaction (PCR) amplification of a *GHI*-specific fragment**

PCR amplification of a 3.2 kb *GHI*-specific fragment has been performed on 65  
unrelated patients. Genomic DNA was extracted from patient lymphocytes by  
30 standard procedures.

Oligonucleotide primers GH1F (5' GGGAGCCCCAGCAATGC 3'; -615 to -599) and GH1R (5' TGTAGGAAGTCTGGGGTGC 3'; +2598 to +2616) were designed to correspond to *GHI*-specific sequences in order to PCR amplify a 3.2kb single genomic DNA fragment containing the human *GHI* gene using the Expand™ high fidelity  
5 system (Roche).

Two separate thin-walled 0.65ml PCR tubes were used for each reaction. The first tube contained 500 nanograms (ng) each primer (GH1F and GH1R), 200μM dATP, dTTP, dCTP and dGTP and 200ng of patient genomic DNA made up to a final volume of  
10 25μl with sterile water. The second tube contained 5μl 10x reaction buffer made up to a final volume of 24.25μl with sterile water. Both tubes were placed on ice for 5 minutes. After this time, 0.75μl of Expand™ polymerase mix was added to the second tube, the contents mixed and transferred to the first tube. The tube was centrifuged for 30 seconds and the reaction mixture overlaid with 30μl light mineral oil (Sigma). The  
15 reaction mixture was then placed in a 480 PCR programmable thermal cycler (Perkin Elmer) set at 95°C. The reaction mix was then amplified under the following conditions: 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 68°C for 2 minutes. For the last 20 cycles, the elongation step at 68°C was increased by 5 seconds per cycle. This was followed by a further incubation at  
20 68°C for 7 minutes and the reaction was then cooled to 4°C prior to further analysis. For each set of reactions, a blank (negative control) was also set up. The blank reaction contained all reagents apart from genomic DNA and was used to ensure that none of the reagents were contaminated.

25 A one-tenth volume (5μl) was analysed on a 1.5% agarose gel to assess whether PCR amplification had been successful before nested PCR was performed. Those samples that had PCR-amplified successfully were then diluted 1 in 100 prior to use for nested PCR.

### Example 3 - Nested-PCR

Nested PCR was performed on the fragments produced in Example 2 to generate, in each case, seven overlapping sub-fragments that together span the entire *GHI* gene. In addition, the Locus Control Region has been PCR-amplified (see Example 5) in all but three patients.

The seven overlapping sub-fragments of the initial 3.2 kb PCR product were PCR-amplified using *Taq* Gold DNA polymerase (Perkin-Elmer). Oligonucleotides used for these reactions are listed in Table 1 together with their sequence locations as determined from the *GHI* gene reference sequence.

A 1µl aliquot of the diluted long (3.2 kb) PCR product was put into a thin-walled 0.2ml PCR tube or into one well of a 96-well microtitre plate. To this was added 5µl 10x reaction buffer, 500ng appropriate primer pair (e.g. GH1DF and GH1DR), dATP, dTTP, dCTP and dGTP to a final concentration of 200µM, sterile water to a volume of 49.8µl, followed by 0.2µl *Taq* Gold polymerase.

The tube or microtitre plate was then placed in a Primus 96 thermal cycler (MWG Biotech) and cycled as follows: 12 min 95°C followed by 32 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 2 minutes. This was followed by further incubation at 72°C for 10 minutes and the reaction was then cooled to 4°C prior to further analysis.

A one-tenth volume (5µl) of the reaction mix was analysed on a 0.8% agarose gel to determine that the reaction had worked before denaturing high-pressure liquid chromatography (DHPLC) was performed on a WAVE™ DNA fragment analysis system (Transgenomic Inc. Crewe, Cheshire, UK). To enhance heteroduplex formation, the PCR product was denatured at 95°C for 5 minutes, followed by gradual re-annealing to 50°C over 45 minutes. Products were loaded on a DNasep column (Transgenomic Inc.) and eluted with a linear acetonitrile (BDH Merck) gradient of 2%/min in a 0.1M triethylamine acetate buffer (TEAA pH 7.0), at a constant flow rate

of 0.9ml/minute. The start and end points of the gradient were adjusted according to the size of the PCR product. Analysis took 6.5-8.5 minutes per amplified sample, including the time required for column regeneration and equilibration. Samples were analysed at the Melt temperatures (TM) determined using the DHPLCMelt software (<http://insertion.stanford.edu/melt.html>) and listed in Table 6. Eluted DNA fragments were detected by an UV-C detector (Transgenomic Inc.).

**Table 6 Oligonucleotide primers used for DHPLC analysis and DNA sequencing**

Fragment	Primer	Sequence (5' to 3')	Position	DHPLC melt temperature
1	GH1DF	CTCCGCGTTCAGGTTGGC	-309 to -292	60°C
	GH1DR	CTTGGGATCCTTGAGCTGG	-8 to +11	
2	GH2DF	GGGCAACAGTGGGAGAGAAG	-59 to -40	63°C
	GH2DR	CCTCCAGGGACCAGGAGC	+222 to +239	
3	GH3DF	CATGTAAGCCCAGTATTTGGCC	+189 to +210	62°C
	GH3DR	CTGAGCTCCTTAGTCTCCTCCTCT	+563 to +586	
4	GH4DF	GACTTTCCCCCGCTGGGAAA	+541 to +560	62°C
	GH4DR	GGAGAAGGCATCCACTCACGG	+821 to +841	
5	GH5DF	TCAGAGTCTATTCCGACACCC	+772 to +792	62°C
	GH5DR	GTGTTTCTCTAACACAGCTCTC	+1127 to +1148	
6	GH6DF	TCCCCAATCCTGGAGCCCCACTGA	+1099 to +1122	62°C
	GH6DR	CGTAGTTCTTGAGTAGTGCGTCATCG	+1410 to +1435	
7	GH7DF	TTCAAGCAGACCTACAGCAAGTTCG	+1369 to +1393	57°C and 62°C
	GH7DR	CTTGGTTCCCGAATAGACCCCG	+1731 to +1752	

#### **Example 4 - Cloning and DNA-Sequencing of *GHI*-specific long PCR fragments**

##### Cloning

DHPLC analysis allowed the identification of DNA fragments containing putative DNA sequence changes. To determine which allele possessed the putative sequence change, *GHI*-specific long (3.2 kb) PCR fragments were cloned into the PCR plasmid cloning vector pGEM-T (Promega). Cloning was accomplished by adding 50ng of *GHI*-specific long PCR fragment to 10ng pGEM-T in the presence of 1x reaction buffer and 1µl (3 units) T4 DNA ligase in a final volume of 10µl. The reactions were

incubated for 16 hours at 10°C. The entire reaction mixture was placed in a 1.5ml tube and cooled on ice. 50µl DH5α competent cells (Life Technologies) were added and the tube left on ice for 30 minutes. The mixture was then heat-shocked for 20 seconds at 37°C and returned to ice for 2 minutes. After this time, 0.95ml of YTx2 medium (16g tryptone, 10g yeast extract, 5g NaCl per litre water) was added and the mixture incubated at 37°C for one hour with shaking. The mixture was then plated out onto pre-warmed agar plates containing 50µg/ml ampicillin, IPTG and X-gal and incubated at 37°C for 16 hours to allow single colonies to grow.

Eight white colonies from each plate were picked and transferred to a second gridded plate. A small amount of each bacterial colony was PCR-amplified using primers GH1DF and GH1DR (see Example 3, Table 6) and the conditions previously described to determine that the *GHI* specific long PCR fragment had been successfully cloned.

Clones that contained the *GHI*-specific long PCR fragment were grown in 2ml YTx2 medium; plasmid DNA was extracted from the bacteria using a Qiagen spin miniprep kit according to the manufacturer's instructions. DNA extracted in this way was quantified by measuring its optical density at 260nm and electrophoresed on a 0.8% agarose gel to verify that the size of the clone was correct. Four of these clones were then sequenced.

#### Automated DNA sequencing

Clones containing the *GHI*-specific long PCR fragment were sequenced with the BigDye sequencing kit (Perkin Elmer) in either 0.2ml tubes or 96-well microtitre plates in a Primus 96 PCR thermal cycler. Oligonucleotide primers used for sequencing were:

GH1S1 (5' GTGGTCAGTGTGGAAGTGC 3': -556 to -537);  
GH3DF (5' CATGTAAGCCAAGTATTTGGCC 3': +189 to +210);  
GH4DF (5' GACTTTCCCCCGCTGTAAATAAG 3': +541 to +560); and  
GH6DF (5' TCCCCAATCCTGGAGCCCCACTGA 3': +1099 to +1122).

1 µg of cloned DNA was sequenced with 3.2 pmol of the appropriate primer and 4 µl BigDye sequencing mix in a final volume of 20 µl. The tube or microtitre plate was then placed in the thermal cycler and cycled as follows: 2 minutes 96°C followed by  
5 30 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. The reaction was then cooled to 4°C prior to purification.

Purification was performed by adding 80 µl 75% isopropanol to the completed sequencing reaction. This was then mixed and left at room temperature for 30 minutes.  
10 The reaction was then centrifuged at 14,000 rpm for 20 minutes at room temperature. The supernatant was then removed and 250 µl 75% isopropanol was added to the precipitate. The sample was mixed and centrifuged for 5 minutes at 14,000 rpm at room temperature. The supernatant was removed and the pellet dried at 75°C for 2 minutes.

15 Samples were then analysed on an ABI Prism 377 sequencer.

#### **Example 5 - Analysis of the growth hormone locus control region**

20 A DNA region approximately 14.5 kb upstream of the human *GHI* gene is known to be involved in the tissue-specific and developmental control of *GHI* gene transcription [Jin *et al. Mol. Endocrinol.* 13: 1249-1266 (1999)]. This is known as the Locus Control Region (LCR) and its DNA sequence was obtained from GenBank (Accession  
25 Number: AF010280). Nucleotide numbering is based on the GH LCR reference sequence (Figure 4). The polymorphic site at position 1192 is marked in bold type and underlined. Part of this region was analysed by PCR and DHPLC.

Two overlapping PCR fragments spanning approximately 400 bp were generated  
30 through the use of novel oligonucleotide primers designed by reference to the available DNA sequence:

Fragment 1 primers were LCR15 (5' GTGCCCCAAGCCTTTCCC 3': 1159-1177)  
and

LCR13 (5' TGTCAGATGTTTCAGTTCATGG 3': 1391-1412); and

fragment 2 primers were LCR25 (5' CCTCAAGCTGACCTCAGG 3': 1346-1363)

5 and LCR23 (5' GATCTTGGCCTAGGCCTCG 3': 1584-1602).

PCR was performed using *Taq* Gold polymerase: 1µl patient genomic DNA was  
placed into a thin walled 0.2ml PCR tube or into one well of a 96-well microtitre plate.

To this was added, 5µl 10x reaction buffer, 500ng of the appropriate primer pair (e.g.  
10 GH1DF and GH1DR), dATP, dTTP, dCTP and dGTP to a final concentration of  
200µM, sterile water to a volume of 49.8µl followed by 0.2µl *Taq* Gold polymerase.  
The tube or microtitre plate was then placed in a Primus 96 thermal cycler (MWG  
Biotech) and cycled as follows: 12 minutes 95°C followed by 32 cycles of 95°C for 30  
seconds, 58°C for 30 seconds and 72°C for 2 minutes. This was followed by a further  
15 incubation at 72°C for 10 minutes and the reaction was then cooled to 4°C prior to  
further analysis.

A one-tenth volume (5µl) was analysed on a 1.5% agarose gel to determine that the  
reaction had worked before denaturing high-pressure liquid chromatography (DHPLC)  
20 was performed. Analysis by DHPLC was performed as described in Example 3 with a  
melt temperature of 61°C.

### Example 6 – *GHI* Gene Mutations and Polymorphisms

25

As can be seen from Table 7, the selection characteristics according to the present  
invention have led to the characterisation and identification of some 24 different and  
novel variants in the *GHI* gene. In Table 7, nucleotide numbering is based on the  
GH1 reference sequence shown in Figure 5, in which the five exons of the human  
30 *GHI* coding sequence are shown in upper case; the translation initiation (ATG) and  
termination codons (TAG) are underlined; the polyadenylation signal is shown in bold  
and is underlined; the 3' UTR boundary is at position +1642; and +1 = transcriptional

initiation site. All numbering of mutational lesions, polymorphisms and oligonucleotide primers referred to in the text (with the exception of the locus control region; see Figure 4) can be related to the *GHI* reference sequence.

5 This sequence is derived from Chen *et al.* (1989) which was accessed through Genbank (Accession Number: J03071). Of 54 patients so far analysed, mutations have been found in 25 of them. All mutations detected were found in the heterozygous state with the exception of patient 37 (homozygous), patients 44, 55 and 57 (compound heterozygous for non-identical lesions) and patients 32, 36 and 48 who  
10 possess either 2 or 3 mutations *in cis* (*ie* on the same allele).

Several novel promoter variants (five single base-pair substitutions, two micro-deletions and an extensive gene conversion event) have been detected plus a common yet novel single base-pair substitution in the obligate AG dinucleotide of an acceptor  
15 splice site. In addition, a common novel variant within the LCR has been found. Further, a total of fourteen novel single base-pair substitutions have been noted within the coding region of the *GHI* gene that change the amino acid encoded. Molecular modelling studies have suggested that they are located in regions of the GH molecule which either interact with the GH receptor or which may influence GH-GH receptor  
20 interactions.

One missense mutation (Lys41Arg) has been found on different haplotype backgrounds in two unrelated patients. This is consistent with recurrent mutation (*i.e.* independent mutation events) at this site. The IVS2 putative splicing mutation was  
25 found in a total of eight alleles in 7 apparently unrelated patients; since two distinct haplotypes are evident, at least two examples of this lesion are likely to be recurrent whilst the remainder may be identical-by-descent. Three examples of the promoter gene conversion event were also noted in this patient sample. These findings are very encouraging in terms of the prospect of the rapid detection of frequent pathological  
30 lesions.



Table 7: Growth hormone deficiency: *GHI* gene mutations and polymorphisms

Patient <sup>a</sup>	Promoter haplotype <sup>b</sup>										Mutation <sup>b,c,d</sup>	Reference	Polymorphisms <sup>b,c,d</sup>
	-168 (T/C)	-75 (A/G)	-57 (G/T)	-31 (ΔG)	-6 (G/A)	-1 (T/A/C)	+3 (G/C)	+16 (A/G)	+26 (A/C)	-59 (T/G)			
1 (3)	T	A	T	G	A	A	G	A	A	T	ND		ND
1 (6)	T	A	G	G	G	T	G	A	A	T	Arg64Gly (AGG → GGG; 799)	Unpublished	IVS4 T → A 1169
2 (3)	T	A	T	G	A	A	G	A	A	T	ND		IVS1 G → A 218
3 (5)	T	A	T	G	A	A	G	A	A	T	Leu163Pro (CTC → CCC; 1442)	Unpublished	IVS1 A → G 141
3 (6)	T	A	G	G	A	A	G	A	A	T	ND		IVS2 A → G 622
4 (1)	T	A	T	G	A	A	G	A	A	T			IVS4 T → C 1196
5 (2)	T	A	G	G	A	A	G	A	A	T	ND		IVS2 A → G 565
5 (4)	T	A	G	-	A	A	G	A	A	T	ND		IVS1 A → G 134
6 (1)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at -1 (702)	Unpublished	IVS1 T → C 179 IVS4 T → A 1169
8 (2)	T	A	T	G	A	A	G	A	A	T	ND		ND
8 (4)	T	A	G	-	A	A	G	A	A	T	ND		Asn152 (AAC → AAT; 1410)
9 (1)	T	G	G	G	G	A	G	A	A	T	ND		IVS4 T → A 1169 IVS4 A → G 1219
9 (3)	T	A	G	G	G	A	G	A	A	T	ND		IVS2 A → G 670 IVS4 T → A 1169 IVS4 T → G 1216 3' UTR T → C 1630
10 (1)	T	G	G	G	G	A	G	A	A	T	Lys41Arg (AAG → AGG; 731)	Unpublished	IVS4 T → A 1169
10 (6)	T	A	T	G	A	A	G	A	A	T	ND		IVS4 T → A 1169
11 (3)	T	A	G	G	G	A	G	A	A	T	ND		IVS2 A → G 580 IVS4 C → T 1101 IVS4 T → A 1169
11 (5)	T	A	G	G	A	A	G	A	A	T	A → G -258	Unpublished	ND
12 (2)	T	A	G	G	G	A	G	A	A	T	A → G -498 G → A -48	Unpublished	IVS4 C → T 1101 IVS4 T → A 1169
12 (4)	T	A	G	G	G	A	G	A	A	T	ND		Glu129 (GAA → GAG; 1341)
13 (1)	T	A	G	G	G	A	G	A	A	T			ND
13 (2)	T	A	G	-	A	A	G	A	A	T	ND		IVS4 C → T 1101
14 (1)	T	A	G	G	G	A	G	A	A	T	ND		IVS4 T → A 1169

15 (1)	T	A	T	G	A	A	G	A	A	G	A	A	T	ND		ND
15 (3)	T	A	G	G	G	A	A	A	A	A	A	A	T	ND		IVS4 T → A 1169
16 (2)	T	A	T	G	A	A	A	A	A	A	A	A	T	ND		ND
16 (7)	T	A	G	G	A	A	A	A	A	A	A	A	T	Lys41Arg (AAG → AGG: 731)	Unpublished	ND
18 (1)	T	A	G	G	A	A	A	A	A	A	A	A	G	C → T -108	Unpublished	IVS1 C → T 188
18 (2)	T	A	G	G	A	A	A	A	A	A	A	A	T	ND		IVS1 A → G 124
19 (2)	T	G	G	G	G	A	A	A	A	A	A	A	T	T → C -418 G → A -156	Unpublished	IVS1 A → T 128
19 (3)	T	A	T	G	A	A	A	A	A	A	A	A	T	ND		IVS4 T → A 1169
20 (2)	T	A	T	G	A	A	A	A	A	A	A	A	T	ND		IVS4 T → A 1169
20 (3)	T	A	G	G	A	A	A	A	A	A	A	A	T	Asp111Asn (GAC → AAC: 431)	Unpublished	IVS4 T → A 1169
22 (1)	T	A	G	G	G	A	A	A	A	A	A	A	T	ND		IVS4 T → A 1169
23 (2)	T	A	G	G	A	A	A	A	A	A	A	A	T	ND		IVS2 G → A 620
23 (8)	T	A	G	G	G	A	A	A	A	A	A	A	T	ND		IVS4 T → A 1169
26 (1)	C	A	G	G	G	T	G	A	A	A	A	A	T	ND		Gly131 (GGC → GGT: 1347)
26 (2)	T	A	G	G	G	A	A	A	A	A	A	A	T	T → C -508	Unpublished	IVS1 T → C 281
27 (2)	T	A	T	G	A	A	A	A	A	A	A	A	T	Ser43Leu (TCA → TTA: 737)	Unpublished	ND
27 (4)	T	G	G	G	A	A	A	A	A	A	A	A	T	ND		IVS4 T → A 1169
28 (4)	T	A	T	G	G	A	A	A	A	A	A	A	T	ND		3' UTR C → T 1659
28 (5)	T	A	G	-	A	A	A	A	A	A	A	A	T	ND		ND
29 (3)	T	A	T	G	A	A	A	A	A	A	A	A	T	ND		ND
29 (3A)	T	A	G	G	G	A	A	A	A	A	A	A	T	ND		IVS1 G → C 286
31 (3)	T	A	G	G	A	A	A	A	A	A	A	A	T	Lys41Arg (AAG → AGG: 731)	Unpublished	IVS4 T → A 1169
32-2 (1)	T	A	G	G	G	A	A	A	A	A	A	A	T	ND		IVS4 A → G 1240
32-2 (2)	T	A	T	G	A	A	A	A	A	A	A	A	T	A → G -177 Ser108Arg (AGC → CGC: 1023) Phe176Ser (TTC → TCC: 1481)	Unpublished	IVS3 T → C 901
32-3 (3)	T	A	G	G	G	A	A	A	A	A	A	A	T	ND		Ins GAAA 251
33 (3)	T	A	T	G	A	A	A	A	A	A	A	A	T	ND		IVS4 T → A 1169
33 (4)	T	?	?	-	G	A	A	A	A	A	A	A	T	Gene conversion (to GH2), max -	Unpublished	ND

34 (3)	C	A	G	G	A	A	G	A	A	A	A	161 to +69, min -86 to -46		IVS4 T → A 1169
34 (4)	T	A	G	G	G	A	G	A	A	A	ND	ND		IVS4 T → A 1169
35 (6)	T	A	G	G	G	A	G	A	A	A	ND	ND		IVS4 T → A 1169
35 (8)	T	G	G	G	G	A	G	A	A	A	ND	ND		IVS4 T → A 1169
36 (3)	T	A	G	G	G	A	G	A	A	A	ND	ND		IVS1 T → C 281 IVS1 T → C 303
36 (5)	T	A	?	G	A	A	G	A	A	A	C → T -309 Del5G -57 to -61 Asp26Val (GAC → GTC: 477)	Unpublished Unpublished Unpublished		ND
37 (1)	T	G	G	G	G	A	G	G	C	T	Acceptor splice site IVS2 G → A at -1 (702)	Unpublished Unpublished		IVS4 T → A 1169
37 (4)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at -1 (702)	Unpublished		IVS4 C → T 1212 IVS4 T → A 1169
39 (1a)	T	G	G	G	G	T	G	A	A	T	ND			IVS4 T → A 1169
39 (3)	T	A	T	G	A	A	G	A	A	T	Thr175Ala (ACA → GCA: 1477)	Unpublished		IVS1 G → C 135
40 (1)	T	A	T	G	G	A	G	A	A	T	ND			ND
40 (5)	T	A	G	G	A	A	G	A	A	T	ND			ND
41 (4)	T	A	G	G	G	A	G	A	A	T				
41 (6)	T	A	?	G	G	A	G	A	A	T	DelG -57 to -61	Unpublished		IVS4 T → A 1169
42 (1)	T	A	G	G	G	A	G	A	A	T	ND			IVS4 T → A 1169
42 (2)	T	A	T	G	A	A	G	A	A	T	ND			ND
43 (4)	T	A	G	G	A	A	G	A	A	T	G → A -343	Unpublished		IVS4 T → A 1169
43 (6)	C	A	G	G	G	T	G	A	A	T	C → T -18	Unpublished		IVS4 T → A 1169
44 (1)	T	G	G	G	G	A	G	A	A	T				3' UTR C → T 1536
44 (3)	T	A	T	G	A	A	G	A	A	T	Seq55Pro (TCG → CCG: 954)	Unpublished		IVS4 T → A 1169
45 (4)	T	G	G	G	G	A	G	A	A	T	ND			IVS1 T → C 281
46 (1)	T	A	G	G	G	A	G	A	A	T	ND			IVS4 T → A 1169
46 (7)	T	G	G	G	G	A	G	A	A	T	ND			IVS4 T → A 1169
47 (4)	T	A	G	G	A	A	G	A	A	T	ND			IVS4 C → T 1101 IVS4 T → A 1169 3' UTR T → C 1648
47 (7)	T	A	T	G	A	A	G	A	A	T				
48 (2)	T	A	G	G	G	A	G	A	A	T	ND			IVS4 T → A 1169

48 (5)	T	G	G	G	G	A	G	A	A	G	Thr-24Ala (ACA → GCA: 69)	Miyata et al (1997) <sup>e</sup> Unpublished	IVS4 T → A 1169
49 (3)	T	A	G	G	A	A	G	A	A	T	Ala155Val (GCA → GTA: 1418) ND	Unpublished	IVS2 T → C 665
53 (1)	T	A	G	G	A	A	G	A	A	T	Ser71Phe (TCC → TTC: 821) ND	Unpublished	IVS2 G → A 676 IVS4 C → T 1182
53 (3)	T	A	T	G	A	A	G	A	A	T			
55 (2)	T	?	?	-	G	A	G	A	A	T	Gene conversion (to GH2), max -161 to +69, min -86 to -46	Unpublished	ND
55 (3)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at -1 (702)	Unpublished	IVS4 T → A 1169
56 (1)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at -1 (702)	Unpublished	IVS4 T → A 1169
57 (1)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at -1 (702)	Unpublished	IVS4 T → A 1169
57 (2)	T	A	G	G	G	A	G	A	A	T	Asp107Gly (GAC → GGC: 1021) ND	Unpublished	IVS4 T → A 1169
58 (1)	T	A	G	G	G	A	G	A	A	T	Gene conversion (to GH2), max -161 to +69, min -86 to -46	Unpublished	IVS4 T → A 1169
58 (4)	T	?	?	-	G	A	G	A	A	T			ND
59 (1)	T	A	G	G	G	A	G	G	A	T	ND		IVS4 C → T 1101 IVS4 T → A 1169
59 (5)	T	A	G	G	G	A	G	A	A	T	ND		IVS4 T → A 1169
60 (2)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at -1 (702)	Unpublished	IVS4 T → A 1169
60 (4)	T	A	G	G	G	A	G	A	A	T	ND		IVS1 T → C 281 IVS4 T → A 1169
61 (1)	T	A	T	G	A	A	G	A	A	T	ND		ND
61 (4)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at -1 (702)		ND
62 (1)	T	A	G	G	G	A	G	A	A	T	ND		IVS4 T → A 1169
64 (7)	T	G	G	G	G	A	G	A	A	T			
64 (8)	T	A	T	G	A	A	G	A	A	T			
65 (1)	T	A	T	G	A	A	G	A	A	T	A → G -248	Unpublished	IVS4 T → A 1169

Key:

IVS: intervening sequence (intron)

ND: no mutation or polymorphism detected

UTR: untranslated region

- \* Patient (clone number)
- <sup>b</sup> Nucleotide numbering based on GH1 reference sequence. At -31, alternative alleles are presence or absence of a G.
- \* Amino acid residue number and substitution, (nucleotide substitution and number based upon *GH1* reference sequence).
- <sup>d</sup> IVS number, nucleotide change, base number
- \* Miyata I, Cogan J, Prince MA, Kamiyo T, Ogawa M, Phillips JA (1997) Detection of growth hormone defects by dideoxy fingerprinting (ddf). *Endocrinol J.* 44: 149-154.

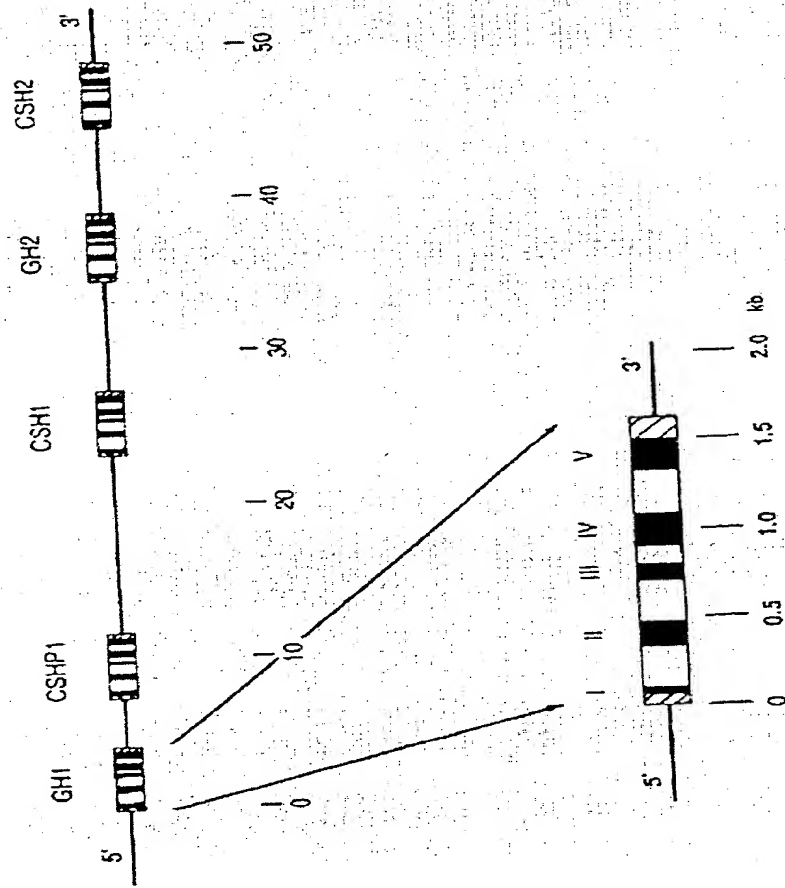


Fig. 1 Human growth hormone gene cluster on chromosome 17q23. The fine structure of the GH1 gene is shown below. The scales are in kilobases (kb)

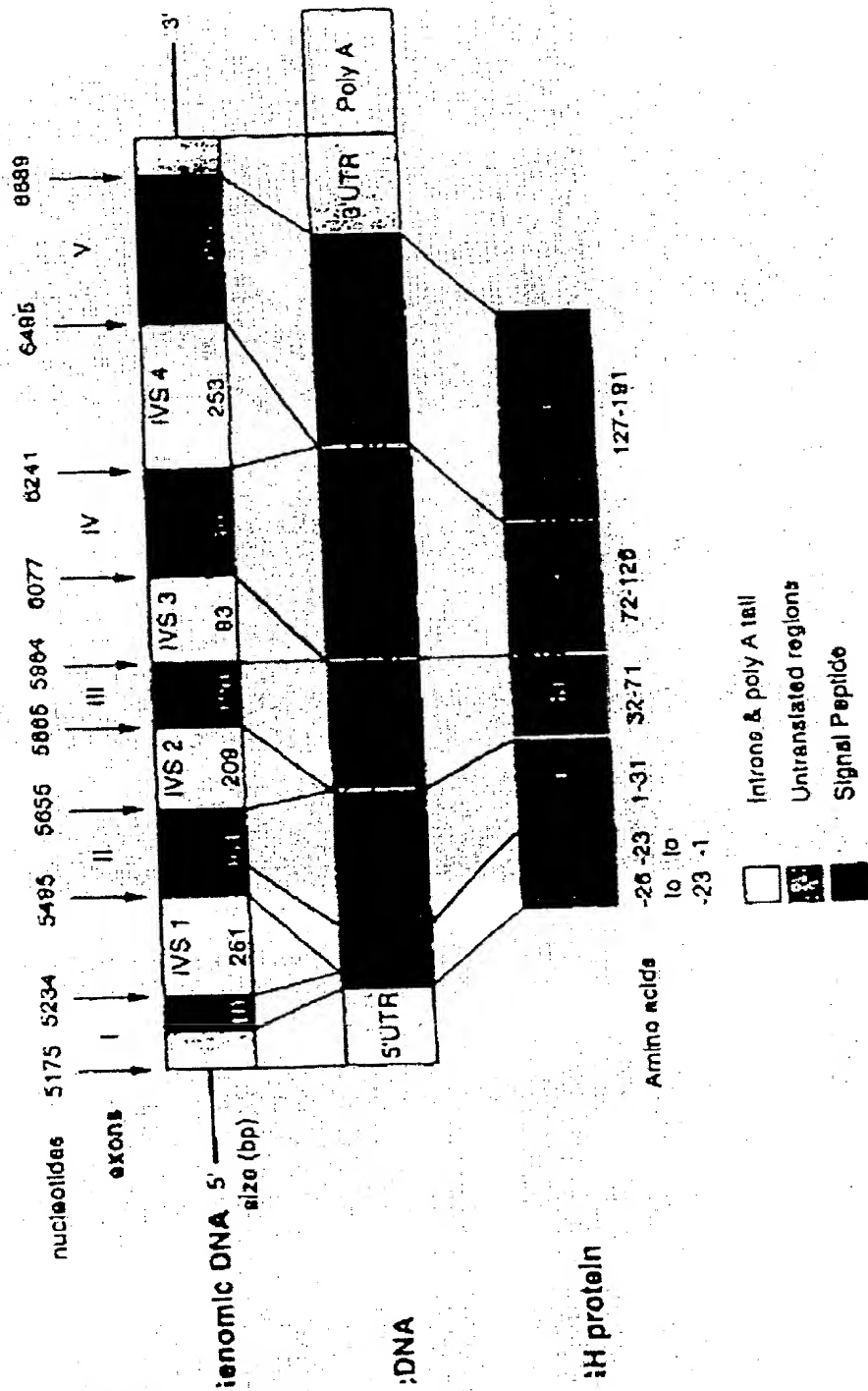
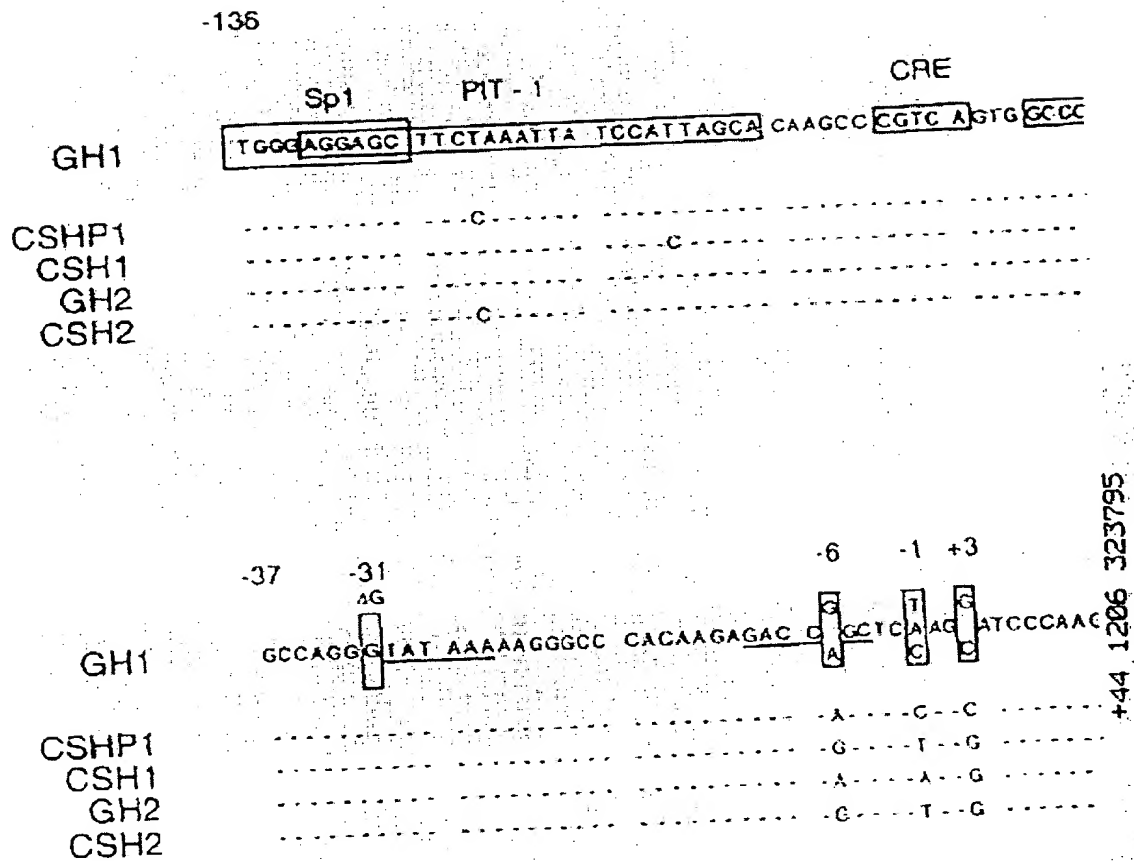


Figure 2



**Fig. 3** Structure of the 5' untranslated region and promoter region of the human *GH1* gene. *Horizontal boxes* denote known, putative or inferred binding sites for transcription factors. *Vertical boxes* indicate polymorphic sites in the human population (data from Giordano et al. 1997; Wagner et al. 1997). The numbering scheme is by reference to the transcriptional initiation site at +1. The TATA box, a Chi-like element and the ATG translational initiation site are *underlined*. The human *GH1* promoter is also aligned with the promoters of the human *CSHP1*, *CSH1*, *GH2* and *CSH2* genes in order to indicate both the extent of homology but also the differences evident between promoters

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**Figure 4**

5  
 1 GATCTTGTCTCAGAAAAACCCAGAAAAACAACAACAAAAAACGCGCTAT  
 51 TGGGCTTATTTCTATTTTACGAAAAATGAGGAACCTTGGTCACTGAGAGGT  
 101 TAAGAATGGGTGTTGCCTCATGGGTGCTGTTGGCATGTGGGGCAGGGAAC  
 151 TCTGCTTTTGCCAGCCAATGCCCCCTCCCGCCCCAGGCACCCACCGGTCATT  
 10 201 GAGACCAGCGGATTGCCCTGATGCATGTGCAGACGCTCCCTCCGTTGGG  
 251 GCAGAGCCACCCTTAGTAGAGAACCCTGGTAACCTGGGGAGGTTTCCTG  
 301 GCTGCTACACTGCTGGGTGAGGACTCAAACCCACGTCCTTCTTAGCAGGT  
 351 CTTTTCCAGTCACAGCAGCCCAGCAGATCCAGGACACCTGGGCTGGAGA  
 401 AGGAGGCAGGGGTCTGAGCTCTTCTTTCCCTGAGGTTCTTCCCCCTCCCCG  
 15 451 GCATCCTAGGGTGGCTGGGGGTGCTGCCTTCTTGCTTCCCCACCCCTG  
 501 AGCCCCCTTGTCCTTTCGCCCCGACCCTGGCCCCCATTCCTTATTTTGATGT  
 551 TGCCTTCTTGAAAGGGAGGTGTGTCGTCAGAAGTCAGAATGTGAAGCCCCG  
 601 GGCAGCCGAGGGCAGAGGCAGGGGTAGGCTTGTGAGGCCTCCTGTTCTGG  
 651 GAAGAAAAGGGCATCATGAGCCCCAAATGGGGCTCAGGGCTGTTGTCACC  
 20 701 AGTGAGGGAACAGACGGCTCAAATGTGACAAATAACCTGCAGGCGGTG  
 751 GGGCCCCCGTGCTCCGCCCCCTCCTGAAAACAGATCCCTGCCCGGCATG  
 801 AATGGGAATGAAGCCCAAAGGCCACATCTGCATCCTTGTCCACGGGATGT  
 851 GTGTGTCTTCCCTCCCTGGAGTGGGAGCTGCTTCTCACTTGTTTTTTTAA  
 901 ATTTTTTTATTTCAATATTTTCTGGGGTACAGGTAGTTTTTTTGGTTACCT  
 25 951 GGATAAGTTCTTTAGTGGTGATTTCTGAGATTTTAGCGCACCTGTACCT  
 1001 GAGGATGGGTGCCAACTGCCCCCACCACCTCCGTGCCCCCTACAGTCC  
 1051 GCTGAGCCCTAGGACCAGCTCCAGAGTAGGCCCCACACCACAGGGTCCAG  
 1101 GCAACACACCTGTGTGAGCATGCACATACACACACACACACACCCCCAC  
 1151 AAACACGTGTGCCCCAAGCCTTTCCAGTTATACCCCCCACTTTGGGGA  
 30 1201 GACACTAGCCCCAAAGTTAATGAAGGACTCTGTGTTAGGGGCTCAGGAA  
 1251 GAGTATTTTCTAAACCATGAGACTCCCAGATTTTGCCCCACTCCCCCGG  
 1301 GTCAGTCTCTCTCCAGCCACCCTCACCAGCATGCGGGCCCATGGGCCTCA  
 1351 AGCTGACCTCAGGTGATGTTTATATTTCTGAGCTGTTTATTCCATGAACT  
 1401 GAACATCTGACAGCTTTTCAGAGAAATGTTTTTTCATTTGGAACATCTGG  
 35 1451 AAACAAGAAAGAACATCTGGGGCTGCCCGGAACGGGCTGTTCCCTCGGATG  
 1501 AAACCTTAACCTCCTGCCCTGACTTCCGTGGCTCAGGTCCTGGCCTGC  
 1551 ACCCCTTGAGAGTGGCCCCACCTTATCAGGTCCGAGGCCTAGGCCAAGA  
 1601 TC

Figure 5

	-700	ctgtttcttg	gtttgtgtct	ctgctgcaag	tccaaggagc	tggggcaata	-651
5	-650	ccttgagtct	gggttcttcg	tccccaggga	cctgggggag	ccccagcaat	-601
	-600	gctcagggaa	aggggagagc	aaagtgtggg	gttggttctc	tctagtggtc	-551
	-550	agtgttgaa	ctgcatccag	ctgactcagg	ctgaccagg	agtcctcagc	-501
	-500	agaagtggaa	ttcaggactg	aatcgtgctc	acaaccccc	caatctattg	-451
	-450	gctgtgcttg	gccccctttc	ccaacacaca	cattctgtct	ggtgggtgga	-401
10	-400	ggttaaaca	gcggggagga	ggaaaggat	aggatagaga	atgggatgtg	-351
	-350	gtcggtaggg	ggtctcaagg	actggctatc	ctgacatcct	tctccgcgtt	-301
	-300	caggttgccc	accatggcct	gcggccagag	ggcaccacag	tgacccttaa	-251
	-250	agagaggaca	agttgggttg	tatctctggc	tgacactctg	tgacacaccc	-201
	-200	tcacaacact	ggtgacggtg	ggaaggga	gatgacaagc	cagggggcat	-151
15	-150	gatcccagca	tgtgtgggag	gagcttctaa	attatccatt	agcacaagcc	-101
	-100	cgtcagtggc	cccatgcata	aatgtacaca	gaaacagggtg	ggggcaacag	-51
	-50	tgggagagaa	ggggccagg	tataaaaagg	gcccacaaga	gaccagctca	-1
	+1	aggatcccaa	ggcccaactc	cccgaaccac	tcagggtcct	gtggacagct	+50
	+51	cacctagcgg	caATGGCTAC	AGgtaagcgc	ccctaaaatc	cctttgggca	+100
20	+101	caatgtgtcc	tgaggggaga	ggcagcgacc	tgtagatggg	acgggggcac	+150
	+151	taaccctcag	gtttggggct	tctgaatgtg	agtatcgcca	tgtaagccca	+200
	+201	gtatttgccc	aatctcagaa	agctcctggt	ccctggagg	atggagagag	+250
	+251	aaaaacaaac	agctcctgga	gcagggagag	tgtgtgacct	ttgctctccg	+300
	+301	gctccctctg	ttgccctctg	gtttctcccc	agGCTCCCGG	ACGTCCCTGC	+350
25	+351	TCCTGGCTTT	TGGCCTGCTC	TGCCTGCCCT	GGCTTCAAGA	GGGCAGTGCC	+400
	+401	TTCCCAACCA	TTCCCTTATC	CAGGCTTTT	GACAACGCTA	TGCTCCGCGC	+450
	+451	CCATCGTCTG	CACCAGCTGG	CCTTTGACAC	CTACCAGGAG	TTTgtaagct	+500
	+501	cttggggaat	gggtgcgcac	caggggtggc	aggaaggggt	gactttcccc	+550
	+551	cgctgggaaa	taagaggagg	agactaagga	gctcaggggt	tttcccgaag	+600
30	+601	cgaaaatgca	ggcagatgag	cacacgctga	gtgaggttcc	cagaaaagta	+650
	+651	acaatgggag	ctggtctcca	gcgtagacct	tgggtggcg	tccttctcct	+700
	+701	agGAAGAAGC	CTATATCCCA	AAGGAACAGA	AGTATTCATT	CCTGCAGAAC	+750
	+751	CCCCAGACCT	CCCTCTGTTT	CTCAGAGTCT	ATTCCGACAC	CCTCCAACAG	+800
	+801	GGAGGAAACA	CAACAGAAAT	CCgtgagtgg	atgccttctc	cccaggcg	+850
35	+851	gatgggggag	acctgtagtc	agagcccccg	ggcagcacag	ccaatgccc	+900
	+901	tccttcccc	gcagAACCTA	GAGCTGTCTC	GCATCTCCCT	GCTGCTCATC	+950
	+951	CAGTCGTGGC	TGGAGCCCGT	GCAGTTCTCT	AGGAGTGTCT	TCGCCAACAG	+1000
	+1001	CCTGGGTGTAC	GGCGCCTCTG	ACAGCAACGT	CTATGACCTC	CTAAAGGACC	+1050
	+1051	TAGAGGAAGG	CATCCAAACG	CTGATGGGGg	tgaggggtggc	gccaggggtc	+1100
40	+1101	cccaatcctg	gagccccact	gactttgaga	gctgtgttag	agaaacactg	+1150
	+1151	ctgccccctt	tttagcagtc	aggccctgac	ccaagagaac	tcaccttatt	+1200
	+1201	cttcatttcc	cctcgtgaat	cctccaggcc	tttctctaca	ccctgaagg	+1250
	+1251	gagggaggaa	aatgaatgaa	tgagaaagg	agggaaacagt	acccaagcgc	+1300
	+1301	ttggcctctc	cttctcttcc	ttcactttgc	agAGGCTGGA	AGATGGCAGC	+1350
45	+1351	CCCCGGACTG	GGCAGATCTT	CAAGCAGACC	TACAGCAAGT	TCGACACAAA	+1400
	+1401	CTCACACAAC	GATGACGCAC	TACTCAAGAA	CTACGGGCTG	CTCTACTGCT	+1450
	+1451	TCAGGAAGGA	CATGGACAAG	GTCGAGACAT	TCCTGCGCAT	CGTGCAGTGC	+1500
	+1501	CGCTCTGTGG	AGGGCAGCTG	TGGCTTCTAG	ctgccccgggt	ggcatccctg	+1550
	+1551	tgacccccctcc	ccagtgcctc	tcctggccct	ggaagtggcc	actccagtgc	+1600
50	+1601	ccaccagcct	tgctcctaata	aaattaagtt	gcatcatttt	gtctgactag	+1650
	+1651	gtgtccttct	ataatattat	gggtggagg	gggtgggtat	ggagcaagg	+1700
	+1701	gcaagtggg	aagacaacct	gtagggcctg	cgggtctat	tcgggaacca	+1750
	+1751	agctggagt	cagtggcaca	atcttgctc	actgcaatct	ccgcctcctg	+1800
	+1801	ggttcaagcg	attctcctgc	ctcagcctcc	cgagtgttg	ggattccagg	+1850
55	+1851	catgcatgac	caggctcagc	taatttttgt	ttttttggta	gagacgggg	+1900
	+1901	ttcaccatat	tggccaggct	ggtctccaac	tcctaattctc	aggtgatcta	+1950
	+1951	cccaccttgg	cctcccaaat	tgctgggatt	acaggcgatga	accactgctc	+2000
	+2001	ccttccctgt	ccttctgatt	ttaaataaac	tataccagca	ggaggacgctc	+2050
	+2051	cagacacagc	ataggctacc	tgccatgccc	aaccgggtggg	acatttgagt	+2100
60	+2101	tgcttgcttg	gcaactgtct	ctcatgcgtt	gggtccactc	agtagatgcc	+2150
	+2151	tggtgaattc	ctgggcctag	ggctgtgcc	gctgcctcgt	cccgtcacct	+2200
	+2201	tctggcttct	tctctccctc	catatcttag	ctgttttcc	catgagaatg	+2250
	+2251	ttccaaattc	gaaatttcta	tttaaccatt	atatatttac	ttgtttgcta	+2300
	+2301	ttatctctgc	ccccagtaga	ttgttagctc	cagaagagaa	aggatcatgt	+2350

+2351 cttttgctta tctagatatg cccatctgcc tggtaaatc tctggcacat +2400  
 +2401 gttacaggca acaactactt gtggaattgg tgaatgcatg aatagaagaa +2450  
 +2451 tgagtgaatg aatgaataga caaaaggcag aaatccagcc tcaaagaact +2500  
 +2501 tacagtctgg taagaggaat aaaatgtctg caaatagcca caggacaggt +2550  
 5 +2551 caaaggaagg aggggctatt tccagctgag ggcaccccat caggaaagca +2600  
 +2601 cccagactt cctacaacta ctagacacat ctgatgctt ttacttctc +2650  
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 +2701 gcacgtccag ttaggtatg cttgtgtac ttcttggtg ttcagagatc +2750  
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 10 +2801 tctcccccg aggtcttccg aggcccttcc tccgtgcctg aacccccctg +2850  
 +2851 acatatcata tggcaaatg aagtgtccaa cgagatatag gaagtgaac +2900  
 +2901 acgatgtaca ctgaaacgtg caatacaaat atgcagcatg aagtcctcg +2950  
 +2951 gttcactaac ccgagctacg ctgggtgctt cttttctacc actttcctta +3000

15

**Figure 6**

Growth hormone 1  
 5 Gene symbol : *GHI*  
 Location : 17q

1 2  
 -26 ATG GCT ACA G↓GC TCC CGG ACG TCC CTG CTC CTG GCT TTT GGC CTG -12  
 Met Ala Thr G ly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu

10 -11 CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT GCC TTC CCA ACC ATT 4  
 Leu Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile

5 CCC TTA TCC AGG CTT TTT GAC AAC GCT AGT CTC CGC GCC CAT CGT 19  
 15 Pro Leu Ser Arg Leu Phe Asp Asn Ala Ser Leu Arg Ala His Arg

2 3  
 20 CTG CAC CAG CTG GCC TTT GAC ACC TAC CAG GAG TTT↓GAA GAA GCC 34  
 Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala

20 35 TAT ATC CCA AAG GAA CAG AAG TAT TCA TTC CTG CAG AAC CCC CAG 49  
 Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln

50 ACC TCC CTC TGT TTC TCA GAG TCT ATT CCG ACA CCC TCC AAC AGG 64  
 Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg

25 3 4  
 65 GAG GAA ACA CAA CAG AAA TCC↓AAC CTA GAG CTG CTC CGC ATC TCC 79  
 Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser

80 CTG CTG CTC ATC CAG TCG TGG CTG GAG CCC GTG CAG TTC CTC AGG 94  
 30 Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg

95 AGT GTC TTC GCC AAC AGC CTG GTG TAC GGC GCC TCT GAC AGC AAC 109  
 Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn

35 110 GTC TAT GAC CTC CTA AAG GAC CTA GAG GAA GGC ATC CAA ACG CTG 124  
 Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu

4 5  
 125 ATG GGG↓AGG CTG GAA GAT GGC AGC CCC CGG ACT GGG CAG ATC TTC 139  
 Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe

40 140 AAG CAG ACC TAC AGC AAG TTC GAC ACA AAC TCA CAC AAC GAT GAC 154  
 Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp

155 GCA CTA CTC AAG AAC TAC GGG CTG CTC TAC TGC TTC AGG AAG GAC 169  
 45 Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp

170 ATG GAC AAG GTC GAG ACA TTC CTG CGC ATC GTG CAG TGC CGC TCT 184  
 Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg Ser

50 185 GTG GAG GGC AGC TGT GGC TTC TAG  
 Val Glu Gly Ser Cys Gly Phe \*

55